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Isolation by differential display and characterization of a tobacco auxin-responsive cDNA *Nt-gh3*, related to *GH3*

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Abstract By use of differential display, we have isolated a new early auxin-responsive cDNA in *Nicotiana tabacum*. *Nt-gh3* had 70% identity with the unique *GH3* sequence isolated in soybean by Hagen et al. [Planta 162 (1984) 147–153] and is thus the first reported cDNA related to this gene until now. *Nt-gh3* mRNA accumulates within a short time after auxin treatment, responds to very low concentrations of NAA (as little as 10^{-9} M) and specifically to active auxins. *Nt-gh3* mRNA is demonstrated to be one of the most relevant early molecular markers of primary auxin response.

Key words: Differential display; Early auxin-responsive gene; *GH3*; Gene expression; Auxin; Tobacco

1. Introduction

The plant hormone auxin is involved in various aspects of plant growth and development like cell elongation, cell division, cell differentiation, root formation and some tropisms [2]. The primary events of auxin signal perception and transduction pathways are still unknown and are the focus of much current work to understand auxin action. Among the primary responses to auxin in plant cells, auxin-induced changes in mRNA expression have been reported. Several early auxin-regulated genes have been identified by differential screening principally in elongating tissues or dividing cells. In dividing suspension cells of *Nicotiana tabacum*, early auxin-regulated genes have been isolated such as *parA*, *parB*, *parC* [3–5] or *pCNT-103*, *pCNT-107* genes [6]. Their expression is enhanced within 10–30 min by exogenous auxin treatment but also by a wide variety of compounds including non-auxin analogues, heavy metals and by various stress conditions [7,8]. In elongating tissues, the first early auxin-responsive cDNA and genes have been isolated from soybean hypocotyls as *Aux22*, *Aux28* [9,10], the *SAUR* genes (small auxin up RNA) [11] and *GH3* [1]. Sequences related to *Aux22* and *Aux28* have been isolated and characterized in plants such as pea [12], mung bean [13] and *Arabidopsis* [14,15]. All of them belong to a large multigene family named *IAA/AUX*. Sequences homologous to the *SAUR* genes have also been isolated and studied in other plant species like mung bean [13] and *Arabidopsis* [16] but to our knowledge no homologue of the soybean *GH3* has yet been characterized. Nevertheless, the soybean *GH3* was demonstrated to be one of the most specifically auxin-regulated genes as only active auxins seem to induce *GH3* transcription [1,17,18]. In addition, activation of *GH3* transcription was reported within 5 min of exogenous auxin application [17]. The steady-state level of the *GH3* transcript is very low in untreated soybean organs, below the detection limit of classical Northern blot analysis [19], but expression studies of the soybean *GH3* have been performed using in situ hybridization [19] as well as transgenic plants expressing the reporter gene *uidA* under the control of the *GH3* promoter [18,20]. Furthermore, studies of the *GH3* promoter have identified three independent AuxREs (auxin-responsive element): E1, D1, and D4, which are auxin-inducible and can function independently of one another [21]. The D1 and D4 domains contain the TGTCTC-AATAAG sequence which has also been found in the promoter of the pea gene, *PS-IAA4/5* [21]. Recently, a yeast one-hybrid system has led to the identification of the first transcription factor (ARF1) that binds to the TGTCTC motif [22]. All these data designated *GH3* as a powerful marker for studying auxin signalling, but they are based on the analysis of a unique soybean gene. We were interested in the isolation of potential new auxin-regulated genes in tobacco and to this end we have applied the differential display reverse transcription polymerase chain reaction method (DD-RT PCR [23]). Using this approach, we have isolated a cDNA showing homology with the soybean *GH3* sequence. We present here the sequence data and the characterization of the expression of the isolated tobacco cDNA, named *Nt-gh3*.

2. Materials and methods

2.1. Plant material

Nicotiana tabacum L. cv Xanthi wild type line XHFD8 seeds were sterilized for 5 min in a solution of NaClO (1.8% active chlorine), washed several times with sterile water, and incubated for 12 h at 4°C. Seeds were then dark-grown for 7 days in Gamborg's medium (Sigma) supplemented with 2% sucrose with moderate shaking (100 rpm) at 26°C.

Tobacco plants were grown from seeds in a greenhouse kept at 22°C. The photoperiod was 9 h for vegetative development and 16 h for induction of flowering. Roots, leaves and stems from plantlets having developed 3–4 leaves were harvested and frozen in liquid nitrogen. Flower buds and flowers were collected from 4-month-old plants.

2.2. Seedling treatments and RNA isolation

For the differential display procedure, as for the cycloheximide and NAA dose-response assays, chemicals were added directly into seedling cultures, which were incubated for the periods indicated in the dark. For the kinetics and the specificity assays of mRNA accumulation, treatments were done in the light. Total RNA was extracted from tobacco plant tissues as described in [24]. When required, poly(A)$^+$ RNA was enriched by one chromatography on oligo(dT) cellulose (Pharmacia) according to the standard method [25].

2.3. Differential display method, reamplification and cloning

100 μg of total RNA was treated with 1 U of DNase RQ1 (Promega) for 30 min at 37°C then ethanol precipitated and resuspended in H_2O DEPC. Starting with 1 μg of DNA-free total RNA, cDNAs

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were synthetized in the presence of 1 μM 5′-T_{12}MG oligonucleotides (where M is either G, A or C) as described in [26]. A two-step procedure of 25 cycles each was performed: the first 25 cycles in the absence of radiolabelled dNTP, the second 25 cycles in the presence of [α-^{33}P]dATP. One tenth of the cDNA was PCR amplified in presence of 10 μM of each dNTP, 3 μM of T_{12}MG, 225 nM of arbitrary primer (O_{15}, Operon), 0.25 U of Taq DNA polymerase (Appligène) with its own buffer containing 1.5 mM $MgCl_2$, in the presence or not of 75 kBq of [α-^{33}P]dATP. After denaturation at 92°C for 3 min, each cycle consisted of 92°C for 45 s, 40°C for 2 min and 70°C for 1 min. The last cycle was followed by a 5 min extension step at 70°C. One sixth of the PCR was mixed with formamide loading buffer and after denaturation at 80°C for 2 min, each sample was resolved on a (6% acrylamide; 7 M urea) gel.

The thin piece of the gel, where the band of interest was located, was recovered as described [23]. The recovered DNA was reamplified using the T_{12}MG and O_{15} primers, by 40 cycles of PCR, all other parameters being as described above. The Northern blot affinity capturing procedure was applied to recover the purified fragment [27]. This fragment was reamplified using modified anchored and O_{15} primers containing *Bam*HI sites. The reamplified product was digested with *Bam*HI, purified and ligated into a *Bam*HI linearized and dephosphorylated BlueScript plasmid.

2.4. Screening of cDNA library, sequencing and sequence analysis

An auxin-induced tobacco seedling cDNA library previously constructed in the group using the Stratagene λZap-cDNA Gigapack II gold cloning kit was screened with the *GO15-21* insert. This DNA fragment was labelled with 1.8 MBq [α-^{32}P]dATP using the Megaprime DNA labelling system (Amersham). Hybridization was done at 60°C in a hybridization buffer containing 5×SSC, 10×Denhardt's solution, 20 mM Na-phosphate buffer pH 7, 7% SDS and 100 μg/ml salmon sperm DNA. The membranes were successively washed in 4×SSC, 1% SDS; 2×SSC, 1% SDS; and 1×SSC, 0.5% SDS for 30 min each and exposed to Kodak X-OMAT film at −80°C. About 450 cDNA positive clones were selected from 500 000 recombinant plaques obtained from the amplified λZap library. Ten positive λZap clones were selected for further purification, through three successive rounds of screening, and excised in vivo following the manufacturer's instructions (Stratagene).

Clones were double-strand sequenced using the ABI prism Dye Terminator kit (Perkin Elmer) and an ABI 373A automated DNA sequencer (Applied Biosystems). Sequence data were analyzed using the Genetics Computer Group (Madison,WI) Version 8.0 software package. Alignments were obtained using Gap, Pileup, Publish and PrettyBox programs of this package.

2.5. Northern blot analysis

Poly(A)$^+$ RNA or total RNA was electrophoresed through 1% agarose gels containing 6% formaldehyde. The RNAs were transferred onto Hybond N nylon membrane (Amersham) in 10×SSC and fixed by UV cross-linking. The Megaprime DNA labelling system (Amersham) was used to label the *GO15-21* insert and the *ubiquitin* probe with 1.8 MBq [α-^{32}P]dATP and the *Nt-gh3* full-length cDNA with both [α-^{32}P]dATP and [α-^{32}P]dCTP. Hybridization conditions were as described in Section 2.2. The hybridization signals were quantified using a PhosphorImager (Molecular Dynamics) and corrected after calibration using the *ubiquitin* signals as references.

3. Results and discussion

3.1. Identification of an auxin-regulated mRNA by differential display

A differential display approach has been developed to identify early auxin-regulated mRNAs in tobacco. Seven-day-old etiolated seedlings, treated or not with 1 μM NAA (1-naphthalene acetic acid) for 30 min, were used to identify differences of mRNA expression appearing in response to auxin within a short time. Among the 120 combinations of primers checked so far, 21 revealed a total of 28 bands (within about 1340 observed) showing a higher accumulation in auxin-treated seedlings compared with mock-treated samples. These

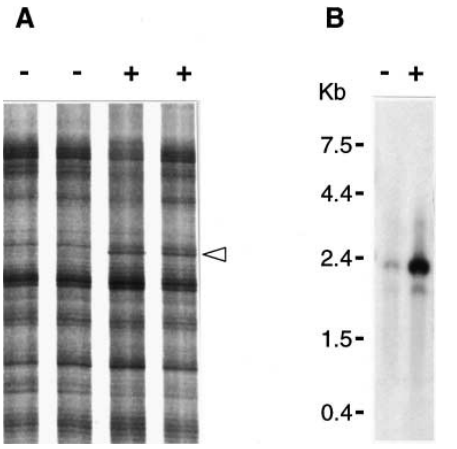

Fig. 1. A: Portion of differential display patterns obtained with RNAs extracted from etiolated tobacco seedlings treated (+) or not (−) with 1 μM NAA for 30 min. The PCR reactions were performed in duplicate. The band GO15, indicated by the arrow, is detected only in treated samples. B: Northern blot of 3 μg poly(A)$^+$ RNAs of untreated (−) and auxin-treated (1 μM NAA for 2 h) (+) seedlings probed with the labelled *GO15-21* insert from the *GO15* PCR product recovery.

bands corresponded potentially to mRNAs which accumulate rapidly after 1 μM NAA treatment in etiolated seedlings. Part of the amplification profile, obtained with the T_{12}MG and O_{15} (Operon) oligonucleotide pair and showing one of the differentially displayed bands (GO15), is presented in Fig. 1A. After reamplification and cloning of the PCR products contained in the GO15 band, several clones were isolated and sequenced. One of them, *GO15-21* (268 nt), hybridized with a mRNA of about 2.2 kbp which accumulates in young tobacco seedlings after NAA treatment (Fig. 1B). The sequence of this insert did not reveal any homology with any known sequence in the databases. The *GO15-21* insert has allowed isolation of several cDNA clones exhibiting similar sequences, by screening of a cDNA library prepared from auxin-treated etiolated tobacco seedlings.

3.2. Sequence analysis of Nt-gh3

The longest cDNA clone sequenced was 2110 bp long and had a potential open reading frame of 1788 bp starting after a 5′-untranslated region of 69 bp and ending at the TGA stop codon at position 1855. As shown in Fig. 2, the 3′-end of the cDNA clone is nearly identical (98.5% identity on 268 nt) to the sequence of the *GO15-21* insert. The open reading frame encodes 595 amino acid residues with a calculated molecular mass of 67.68 kDa and an isoelectric point of 6.58. Both Chou and Fasman [28] and Kyte and Doolittle [29] algorithms predict that the putative protein is mainly composed of hydrophilic residues indicating that it is likely to be a soluble protein. No leader peptide signal, nuclear localization sequence or other particular recognition motif could be identified.

A computer search of databases has revealed that the deduced amino acid sequence shares 74% identity and 79% similarity with the predicted amino acid sequence of the soybean auxin-responsive transcript *GH3* [18] and 39% identity and 46% similarity with a translated region of an EST from *Arabidopsis* [30] (Fig. 3). For consistency in nomenclature, we named the tobacco cDNA sequence *Nt-gh3*, according to the soybean sequence and the plant species from which it

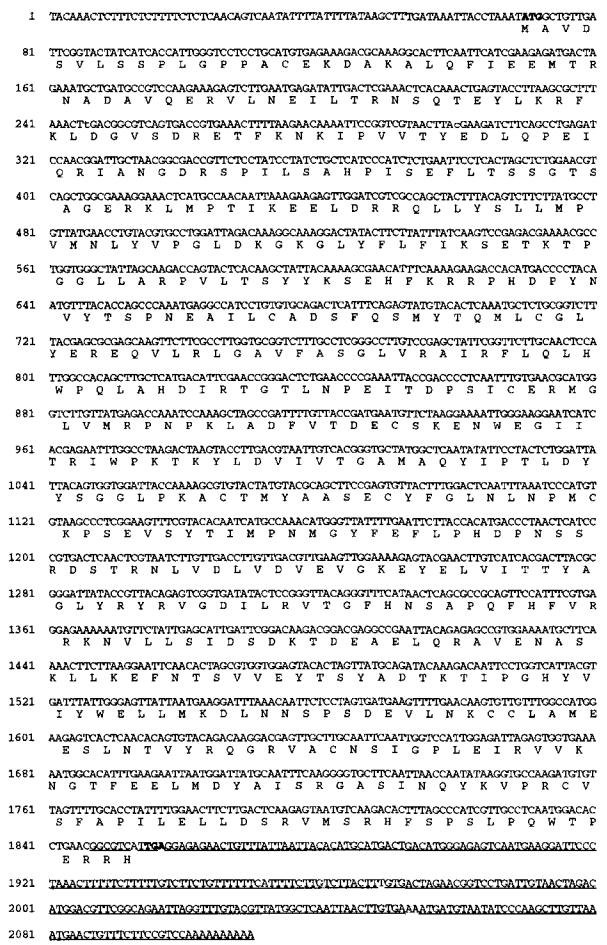

Fig. 2. Nucleotide and deduced amino acid sequences of *Nt-gh3* cDNA. The underlined nucleotides indicate the overlap with the PCR fragment *GO15-21*. The predicted ATG codon and stop codon are indicated in bold characters.

was isolated (*Nicotiana tabacum*). For the same reason, the soybean (*Glycine max*) sequence is named *Gm-gh3*. The identity within the two predicted proteins, Nt-gh3 and Gm-gh3, is distributed along the whole sequence but contains several stretches of highly conserved amino acids. As tobacco is not closely related to the legumes, these conserved regions should designate domains of structural or functional importance for the GH3 proteins.

3.3. Rapid Nt-gh3 mRNA accumulation in response to NAA and cycloheximide

To examine the accumulation kinetic of *Nt-gh3* mRNAs, etiolated tobacco seedlings were treated with 3 μM NAA for periods up to 24 h. Using total RNAs, the steady-state level of *Nt-gh3* transcripts is undetectable in etiolated seedlings prior to the auxin treatment (Fig. 4A). A slight increase of the hybridization signal was observed after 10–15 min incubation with 3 μM NAA. Within 2 h, the half-maximal response was reached and a maximum was observed after 6 h of auxin treatment, the hybridization signal then decreasing progressively. These data indicate that the accumulation of *Nt-gh3* transcripts is rapidly but transiently increased in response to auxin stimulation. The accumulation kinetics of *Nt-gh3* mRNAs is consistent with the results reported for *Gm-gh3* after slot blot analysis [11] and in vitro nuclear transcription assays [17] on elongating sections of soybean hypocotyl or on soybean plumules treated with 50 or 100 μM 2,4-D, respectively. Hagen and Guilfoyle [17] demonstrated that the *Gm-gh3* gene was transcriptionally induced shortly after auxin addition to seedlings or excised plant organs. Considering the accumulation kinetics of *Nt-gh3* in response to auxin treatment in intact tobacco seedlings, we can postulate that the observed increase in *Nt-gh3* mRNA level is mainly due to transcriptional activation of the corresponding tobacco gene as for *Gm-gh3*.

Incubation of etiolated tobacco seedlings with 50 μM of the

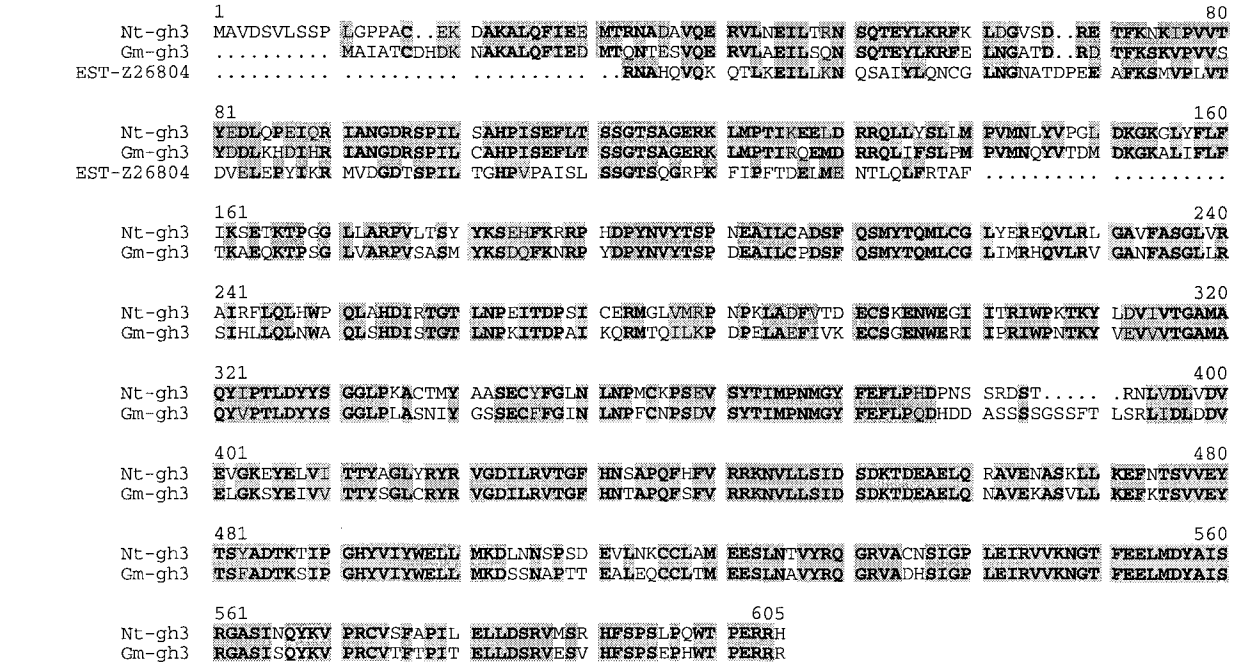

Fig. 3. Amino acid sequence comparison between the deduced amino acid sequence of *Nt-gh3*, the soybean *Gm-gh3* [18] and an *Arabidopsis* EST sequence, ST-Z26804 [30]. Identical amino acids are indicated in bold characters and amino acid similarities appear in shaded areas.

protein synthesis inhibitor cycloheximide (CHX) for 2 h resulted in accumulation of high levels of *Nt-gh3* mRNAs, similar to those reached after auxin incubation (Fig. 4B). When CHX treatment was combined with NAA, *Nt-gh3* mRNAs accumulated more than after incubation with CHX or NAA alone. This result indicates that de novo protein synthesis is not required to observe the increase of *Nt-gh3* mRNAs, classifying *Nt-gh3* among the primary responsive genes to the auxin signal. The effect of CHX alone has been reported for other early auxin-regulated genes such as *SAUR* [31] and *IAA/AUX* [32] and could result from either a stabilization of the mRNA [31] and/or transcriptional activation [32]. CHX has been demonstrated to be ineffective upon the accumulation of *Gm-gh3* but did not inhibit the induction of transcription by auxins [17,31]. This difference of response may reflect distinct control of *Nt-gh3* and *Gm-gh3* expression at the transcriptional or mRNA levels.

3.4. Sensitivity and specificity of the Nt-gh3 response

The effect of different NAA concentrations, from 10^{-10} M to 10^{-4} M, was investigated for the expression of *Nt-gh3*. Fig. 5A shows the dose response for intact etiolated seedlings after 2 h of auxin exposure. Accumulation of *Nt-gh3* transcript was detected with as little as 10^{-9} M NAA, exhibiting a 2.5-fold increase compared to seedlings incubated in the absence of auxin. *Nt-gh3* mRNA continued to accumulate after NAA treatments up to 10^{-5} M but the higher concentration tested

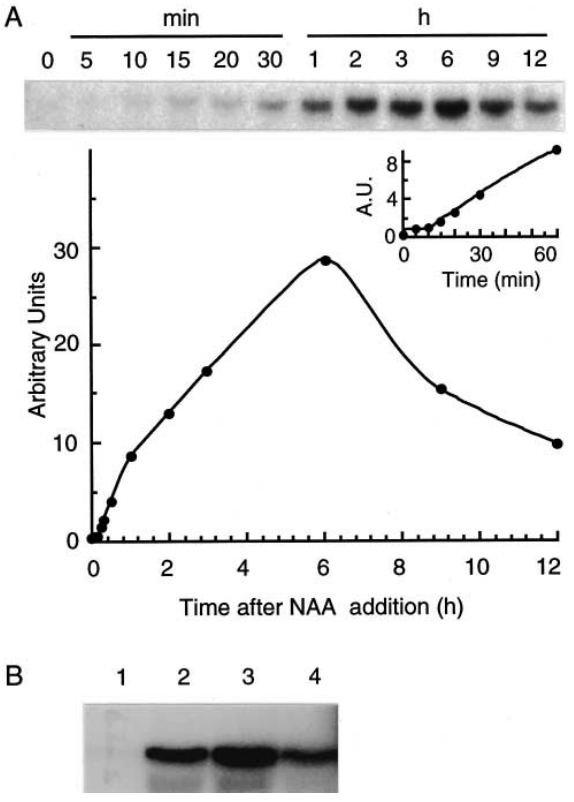

Fig. 4. A: Time course of expression of *Nt-gh3* in response to 3 μM NAA in etiolated tobacco seedlings. 30 μg of total RNA for each point was subjected to RNA gel blot analysis. B: Effects of cycloheximide and auxin on *Nt-gh3* mRNA accumulation in tobacco seedlings. Etiolated seedlings were either mock-treated (1), treated with 50 μM cycloheximide for 2 h (2), treated with 3 μM NAA for 90 min following (3) or not (4) 30 min of 50 μM CHX pre-treatment. 30 μg of total RNA were used for Northern blot analysis.

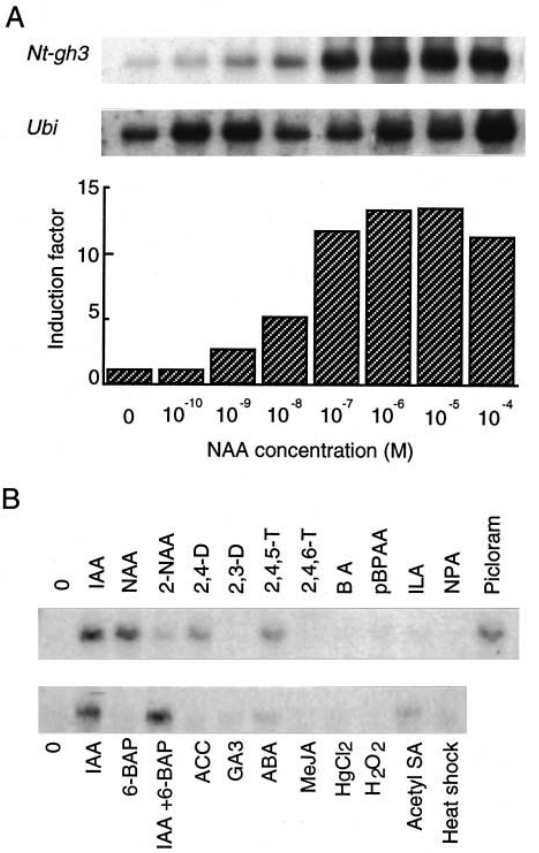

Fig. 5. A: NAA dose response of *Nt-gh3*. Seven-day-old tobacco seedlings were incubated for 2 h with NAA at the concentrations indicated. 2 μg of poly(A)$^+$ RNA was used for Northern blot analysis and hybridized successively with the *Nt-gh3* and *ubiquitin* probe. B: Expression of *Nt-gh3* in response to various effectors. Young etiolated tobacco seedlings were mock-treated (0) or treated for 2 h with 3 μM of the following compounds: indole-acetic acid (IAA); α-naphthalene acetic acid (NAA); β-naphtalene acetic acid (2-NAA); 2,4-dichlorophenoxyacetic acid (2,4-D); 2,3-dichlorophenoxyacetic acid (2,3-D); 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); 2,4,6-trichlorophenoxyacetic acid (2,4,6-T); benzoic acid (BA); *para*-bromophenylacetic acid (pBPAA); indolelactic acid (ILA); 1-*n*-naphthylphthalamic acid (NPA); 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (picloram). The other compounds tested were at the concentrations indicated below: 10 μM 6-benzoaminopurine (6-BAP), 1 mM 1-aminocyclopropane-1-carboxylic acid (ACC), 10 μM gibberellic acid (GA3), 10 μM *cis*-abscissic acid (ABA), 10 μM methyl jasmonate (MeJA), 0.1 mM mercury chloride ($HgCl_2$), 1 mM hydrogen peroxide (H_2O_2), 1 mM acetylsalicyclic acid (Acetyl SA), at 42°C for 1 h then 28°C for 1 h (heat shock). 30 μg of total RNA was subjected to RNA gel blot analysis.

was less effective in increasing the mRNA level, suggesting an inhibitory effect for supra-optimal concentrations. The relative effect of different auxins, structurally related compounds, plant growth substances and other chemicals has also been investigated on the accumulation of *Nt-gh3* mRNA (Fig. 5B). A significant increase of the mRNA level was observed after treatment with the active natural or synthetic auxins whereas none of the other treatments affected *Nt-gh3* expression, revealing a specific action of active auxin compounds on the regulation of *Nt-gh3*. All the auxins were applied to the seedlings at 3×10^{-6} M for 2 h, in which conditions the natural auxin IAA and the synthetic auxin NAA are more efficient than the other synthetic auxins 2,4-D, 2,4,5-T and picloram in inducing *Nt-gh3* accumulation. In addition, we

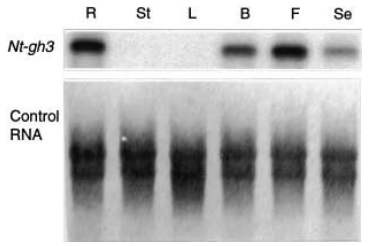

Fig. 6. Organ-specific expression of *Nt-gh3* in tobacco. 8 μg poly(A)$^+$ RNA extracted from various organs was subjected to RNA gel blot analysis. Roots (R); stems (St); leaves (L); flower buds (B); flowers (F); seedlings (Se). The methylene blue staining of poly(A)$^+$ RNA after blotting onto membrane is presented (control RNA).

observed that at least 10^{-7} M 2,4-D was needed to detect an increase of *Nt-gh3* mRNA (data not shown) whereas 100 times less NAA was necessary to obtain the same response. These differences of response could reflect differences of auxin accumulation between IAA, NAA and the other auxins but also differences of sensitivity of *Nt-gh3* to these distinct chemicals.

Different auxins have been reported to be more or less effective in inducing *Gm-gh3* in soybean or transgenic tobacco but most of the experiments have been done at high concentrations of auxins (5×10^{-5} or 10^{-4} M) where an inhitory effect can already interfere. At the concentration of 10^{-4} M 2,4-D and 2,4,5-T were shown to be more effective than NAA than IAA in inducting *Gm-gh3* [17]. For *Nt-gh3*, we can classify the efficiency of the auxins in the opposite order but our comparison was performed at lower concentrations. Dose responses to exogenous auxin have also been described for *Gm-gh3* by nuclear run-on [17], protein accumulation [33] and more recently GUS assays in transgenic tobacco expressing the *uidA* gene product under the control of the *Gm-gh3* promoter [18]. Low concentrations of NAA (10^{-7}–10^{-8} M) were shown to induce the transcription of *pGm-gh3* in tobacco seedlings but 10–100-fold higher concentrations of 2,4-D were required to measure an induction of *Gm-gh3* in soybean. In summary, both *Nt-gh3* and *Gm-gh3* respond only to active auxins but a more precise comparison of their inducibility remains difficult as the plant material, the auxin compounds and the concentrations used were not identical.

3.5. Basal mRNA expression of Nt-gh3 in tobacco plants

The steady-state level of *Nt-gh3* mRNA was examined in different organs of mature tobacco plants (Fig. 6). By using 8 μg poly(A)$^+$ RNA and a double labelled cDNA probe, it was possible to detect *Nt-gh3* mRNA in tobacco organs as well as in etiolated seedlings. The highest expression was observed in roots and mature flowers but, considering the particular conditions used to perform the Northern blot analysis, the steady-state level of *Nt-gh3* in these organs was still low. No hybridization signal was detected in stems and mature leaves.

In soybean, *Gm-gh3* transcript level was undetectable by Northern blot analysis in soybean hypocotyl [11]. By in situ hybridization, it has been shown that *Gm-gh3* mRNAs were expressed at low levels in soybean roots and at highest levels in floral organs [19]. The expression patterns of the tobacco and soybean *GH3* appear to be similar in the absence of exogenous treatment.

Using the mRNA differential display method, we have isolated a cDNA tobacco clone related to the soybean *GH3* gene (*Gm-gh3*). The characterization of *Nt-gh3* revealed several similarities with *Gm-gh3*; in particular, both respond only to active auxins within a short time of about 10–15 min after treatment with exogenous auxins. However, the regulation of *Nt-gh3* also differs from *Gm-gh3* with regard to the CHX effect and the sensitivity to auxin response. Hagen et al. [18] point out that *Gm-gh3* is part of a small multigene family in soybean and that other *Gm-GH3*-like mRNAs are also expressed. We have to consider the possibility that *Nt-gh3* could be the homologue of another member of the *Gm-GH3* family. The isolated *Nt-gh3* cDNA is clearly one of the most interesting markers of the early auxin response and will be valuable in further studies of auxin signalling in tobacco.

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